

The NO world for plants: achieving balance in an open system

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ABSTRACT

Nitric oxide (NO) is a free radical that had been known for many years simply as a toxic air pollutant. The discovery of enzymatic NO production in many living organisms has established a new paradigm: NO being an essential molecule endogenously produced in the cells. In plant science it has been suggested that NO acts as a plant hormone equivalent to ethylene; that is, as a gaseous signal transmitter. Even after experiencing such a scientific breakthrough, however, researchers may still feel difficulty in exploring plant NO signalling systems with conventional approaches. A major difference between plants and animals is that the growth and development of plants is closely linked to the surrounding environment where NO levels vary according to biotic and abiotic activities. This fundamental difference may make the NO-signalling network system of plants larger and more complicated than that of vertebrates. This review intends to show prospects for the future of NO signalling research in plants by introducing a holistic concept to aid in the exploration of complicated systems such as the plant-environment system. Furthermore, the novel ONS hypothesis is proposed to encompass the complexity and simplicity of NO in chemistry, biochemistry and physiology.

Key-words: arginine pathway; holistic science; integrative biology; nitration; nitrite pathway; ONS hypothesis; reactive nitrogen species; reactive oxygen species.

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; O_2^- , superoxide; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSS, reactive sulphur species.

INTRODUCTION

Nitric oxide (NO) is one of the simplest bioactive molecules. For plant biologists, NO had long been appreciated only as a harmful air pollutant. Until the 1990s, in fact, most of our interests oriented toward research on the physiological impact of nitrogen oxides ($NO_x = NO + NO_2$) on plants

and animals. The discovery of the enzymes that produce NO with NADPH and L-arginine have entirely changed our recognition of NO: from just an air pollutant to an essential signalling molecule. Such a historical big wave in science, of course, has led plant biologists to the idea that plants may possess a NO signalling system similar to that found in animals (for historical background, see Yamasaki 2004). The search for mammalian-type NO synthase (NOS) in plants was the primary interest at that time. Indeed, much biochemical research did suggest that plant cells seem to include a mammalian-type NOS activity (Delledonne *et al.* 1998). However, there has been no evidence for the presence of a plant NOS structurally similar to the mammalian one (Wendehenne, Lamotte & Pugin 2003; Corpas, Barroso & del Rio 2004). Although two enzymes that show low sequence homology to mammalian NOSs (eNOS, iNOS, nNOS) have recently been identified (Chandok *et al.* 2003; Guo, Okamoto & Crawford 2003), the physiological significance of those enzymes in plant NO signalling systems is still a subject to be explored (Wendehenne *et al.* 2003; He *et al.* 2004).

It is now a consensus for plant biologists that NO is an important gas molecule that is comparable with the plant hormone ethylene (Leshem 2000; Guo *et al.* 2003). NO has been reported to participate in many physiological phenomena: germination (Beligni & Lamattina 2000; Simontacchi, Jasid & Puntarulo 2004), induction of cell death (Pedroso, Magalhaes & Durzan 2000), pathogen response (Clarke *et al.* 2000; Chandok *et al.* 2003), stomata regulation (Neill, Desikan & Hancock 2003; Sakihama, Murakami & Yamasaki 2003a), and photosynthesis regulation (Takahashi & Yamasaki 2002). In spite of its potential significance, however, plant researchers and medical researchers alike may feel confusion and encounter unique difficulties with investigations of NO. Why does this happen? In biochemistry and molecular biology, major target molecules are stable, big and unique in structure such as proteins, sugars, lipids DNA or RNA. The nature of the NO gaseous radical is opposed to these conventional biomolecules: unstable, small, simple, and ubiquitous. Owing to these properties conventional approaches are generally not applicable for investigations into signalling and other biological functions of NO. In this context, we may need a new paradigm for NO research as seen in the central dogma of molecular biology (DNA → RNA → protein) established in the early 1970s.

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Because there are many review papers that cover the up-to-date progress in NO plant biology especially in the field of NO signalling, this review will not give a comprehensive overview of current plant NO research. Instead, some prospects for addressing the complexity of plant NO signalling systems by revealing the underlying simplicity are presented. Wishing to be of help toward the future plant NO research, I propose the ONS hypothesis, a novel concept for a better understanding of the NO world in plants.

NO PRODUCTION MECHANISM IN PLANTS: SIMPLE OR COMPLEX

Figure 1 summarizes the major NO-producing pathways in plants. Until now, two distinct pathways in plants have been elucidated: the arginine pathway and the nitrite pathway (Fig. 1). The former can be mediated by NO synthase (NOS) with the substrates L-arginine, O_2 , and NADPH. As the result of this reaction, the product L-citrulline is produced along with NO. Competitive inhibition should occur when analogues of L-arginine such as N^G -nitro-L-arginine methyl ester (L-NAME) are present. Two distinct enzymes in plants have been shown to possess NOS activity – the production of NO in the presence of L-arginine, NADPH and O_2 (Chandok *et al.* 2003; Guo *et al.* 2003). The findings

of NOS unique to plants may provide explanations for previous studies that reported NOS-like activity in plants. Many bacteria have recently been found to possess the NOS gene and proteins but the biological function of bacterial NOS is largely unknown (Cohen & Yamasaki 2003). Kers *et al.* (2004) have suggested that the primary role of bacterial NOS might not be for producing NO but rather for synthesizing specific molecules. They have shown that the bacterium *Streptomyces turgidiscabies* needs NOS to synthesize the phytotoxin thaxtomin A, a nitrated dipeptide that is required for plant pathogenicity (Kers *et al.* 2004). This finding reminds us to be more cautious about the possibility that the physiological function of plant NOS may not be only NO production.

The enzymatic mechanism for NO production by NOS is complicated and the reaction needs many cofactors (e.g. B_4H , FAD, or Ca^{2+}) (Wendehenne *et al.* 2003). In contrast to the complexity of the arginine pathway, the mechanism of NO production from nitrite is quite simple. NO can be produced through one electron reduction of nitrite by chemical as well as enzymatic routes (nitrite pathway). In the chemical route of the nitrite pathway, NO production is virtually negligible at neutral and alkaline pHs because the reaction requires HNO_2 but not NO_2^- (Yamasaki 2000; Sakihama, Nakamura & Yamasaki 2002). Therefore, chem-

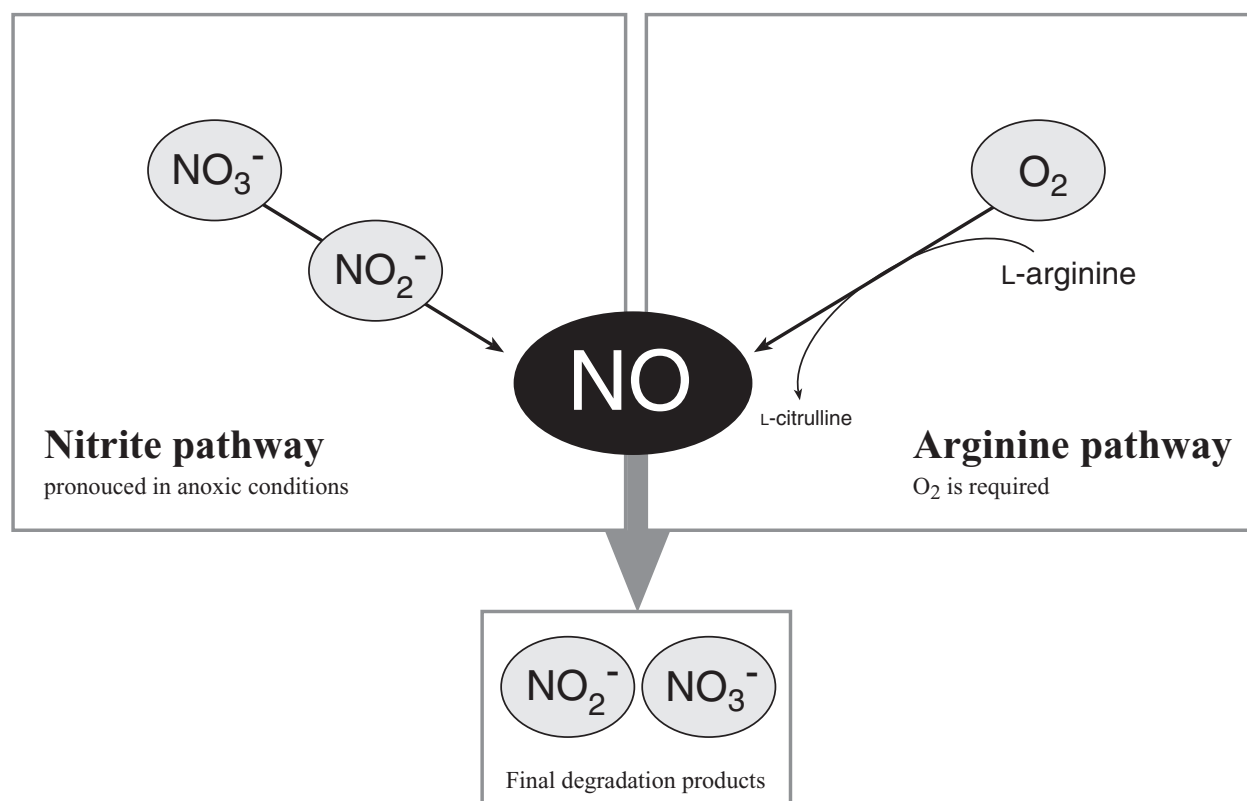


Figure 1. Two pathways for NO production in plant systems. There are two major mechanisms for NO production in plants: the nitrite pathway and the arginine pathway. The nitrite pathway is a simple way to produce NO through one electron reduction of nitrite. This reaction also occurs with many enzymes that include a redox domain. Assimilatory nitrate reductase (NR) has been firstly confirmed as such an enzyme. The other is the arginine pathway that can be catalysed by nitric oxide synthase (NOS) with L-arginine, NADPH and O_2 . The fundamental difference in the mechanism is that the arginine pathway requires O_2 in the reaction whereas the nitrite pathway does not.

ical NO production via the nitrite pathway is pronounced only in acidic compartments or tissues under healthy conditions. However, wounding by physical as well as biotic factors (such as grazing by herbivores) that results in physical destruction of cell compartments can induce NO production by mixing nitrite (cytosol), acid (vacuole) and reductants (chloroplast), a potential mechanism for inter-organ or inter-plant signal transmission by NO (Yamasaki 2004).

Nitrate reductase (NR) has been shown to have NO-producing activity *in vitro* (Yamasaki, Sakihama & Takahashi 1999; Yamasaki & Sakihama 2000) as well as *in vivo* (Rockel *et al.* 2002; Lea *et al.* 2004). NR is a key enzyme of nitrate assimilation metabolism and nitrate reduction is known as the rate-limiting step in the course of the metabolism. NO production by NR was reported in the 1980s, but the reaction was presumed to be unique to legume NR (Yamasaki 2000). It is now evident that all NRs potentially produce NO from nitrite in the presence of NAD(P)H. Involvement of NR in stomata regulation (Neill *et al.* 2003; Sakihama *et al.* 2003a), germination (Simontacchi *et al.* 2004), and pathogen response (Yamamoto *et al.* 2003) have been reported. It seems that NR behaves like a NOS in plants.

The fundamental differences between the nitrite and arginine pathways are: (1) the nitrite pathway can proceed even in the absence of an enzyme; and (2) the presence of O₂ is essential for the arginine pathway, whereas the nitrite pathway does not require O₂ (its activity is even higher in anoxic or hypoxic conditions). It is very important to be reminded that in the nitrite pathway, assimilatory NR is not the only enzyme involved in nitrite-dependent biological NO production. Although we must wait for biochemical evidence, there are many enzymes with redox domain(s) that could potentially reduce nitrite to form NO (Corpas *et al.* 2004). Bacterial denitrification (as well as nitrification) processes can be attributed to the nitrite pathway.

On either pathway, nitrite or nitrate should eventually be produced as the final degradation product of NO. The complexity of the relationship between substrates and products in NO metabolism brings difficulties in identifying the molecules directly involved in the signalling systems; its degradation product (nitrite or nitrate) but not NO itself may display a signalling activity or *vice versa*. Thus, we always should be careful when interpreting experimental results – whether it is really due to a direct effect of NO or due to secondarily produced reactive nitrogen species (RNS), nitrite or nitrate? It has been reported that plants possess abundant unidentified nitrogen compounds (UNs) that may be the reaction products of NO or RNS (Morikawa *et al.* 2004; Sakamoto *et al.* 2004). Because the lifetime of NO is short in atmospheric conditions (e.g. a few seconds), the actual molecules that transmit the NO signal might be other than NO. It is worthwhile to consider reaction products (nitrated or nitrosylated compounds) as potential agents for transmitting the NO signal (Sakihama *et al.* 2003b; Yamasaki 2004). This might also be the case for NO communication in plant–microbe interactions in soils.

BALANCE OF THE OPPOSITES: AN ULTIMATE ANSWER

When we look back at the history of how NO came to be recognized in science, we are reminded that there have always existed two extremes for any given bioactive molecules: good or bad; protective or toxic; friend or foe. The apparent diametrically opposed roles for NO lead to confusion even for current researchers. To account for such dualism, we have applied the Yin–Yang principle (see detail, Yamasaki 2004) which emphasizes that the balance is important. Balance science, a holistic approach for resolving problems in complicated system, can be found in many oriental philosophies, such as the Yin–Yang theory in China, Dosha of Ayurveda in India, Bushido in Japan. Although these oriental philosophies and western science may be mutually exclusive, the concepts could help in the understanding the fundamentals of simple and abundant molecules such as NO or O₂.

EVOLUTIONAL BACKGROUND FOR THE OPPOSITES: SIMPLE TO COMPLEX

The dualism (good and bad) of NO in effects may be better explained by introducing an evolutionary aspect rather than a philosophical argument. Figure 2 intends to present how biological relationships change in the process of evolution (Yamasaki 2004). In an early stage, hostile invaders (element A) just cause harmful effects to living organisms (element B): attack (stage 1). Later, living organisms evolve protecting mechanisms against harmful invaders: tolerance (stage 2). After the acquisition of the protecting mechanism, invaders become friends at last and may have a new function for mutual benefits via tolerance stage: new function (stage 3). Finally, living organisms may actively produce the element inside for new functions: active production (stage 4). We can apply this sequential change in relationship to interactions between living organisms (infection, parasitism, symbiosis) and inorganic molecules such as O₂ or NO (oxidative destruction, antioxidant systems, enzymatic production). The versatility of NO can also be explained well by the above concept. Inside their cells therefore evolutionally advanced organisms like vertebrate animals and vascular plants could include such a long history of changes in the relationship to NO, providing a reason for the presence of opposites.

INTERPLAY OF NITROGEN AND OXYGEN RADICALS: ANOTHER CAUSE OF THE COMPLEXITY

Just as reactive oxygen species (ROS) do not represent a specific molecule, NO, ONOO[−] and their reaction products are frequently referred to as reactive nitrogen species (RNS). In general, ROS production in the cells is higher in atmospheric conditions due to aerobic respiration of mitochondria. In contrast, oxygen availability is low in hypoxic conditions that plant roots may experience, especially dur-

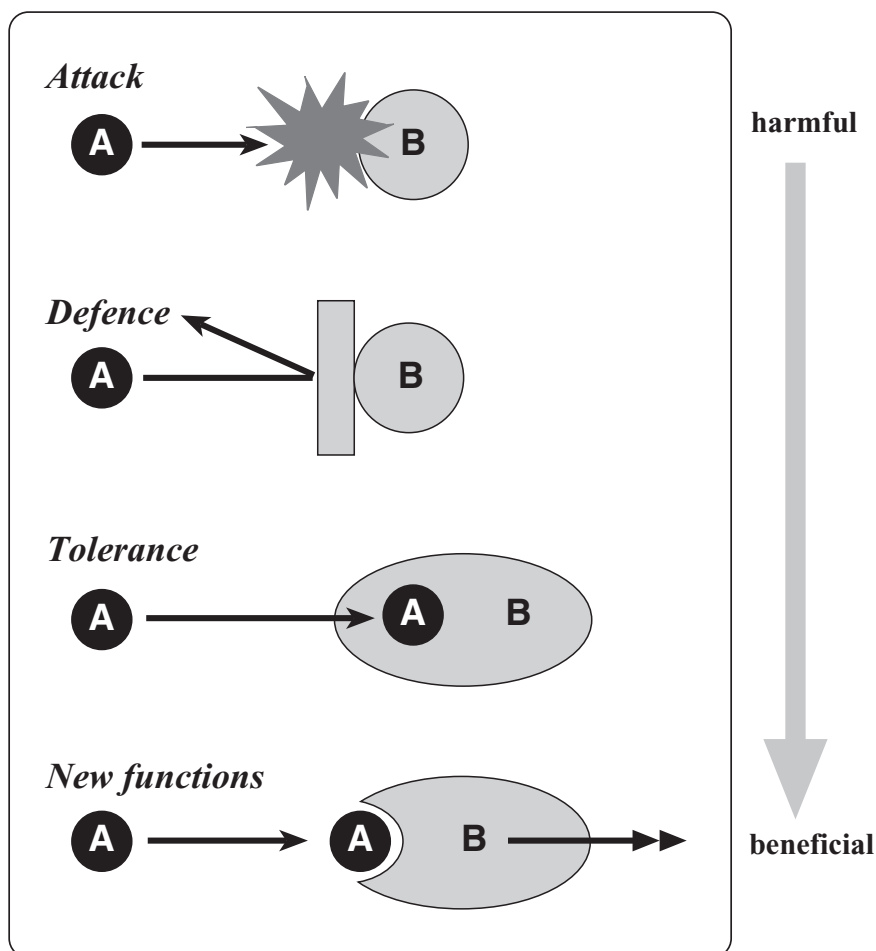


Figure 2. Evolutional change in a dual relationship. This scheme represents conceptual steps suggesting how living organisms have evolved to cope with unfavourable exogenous element(s). Suppose the element A is harmful to the element B. At the beginning, B suffers attack by A (stage 1). Then, B develops protection mechanisms and further evolves tolerance mechanisms (stage 2). Finally, the harmful elements play new functions for B (stage 3). Eventually, B is able to actively produce A to utilize it. This concept can account for the dualism of NO in its effects on plants: harmful and beneficial in the same cells. Cytotoxicity of NO may be a reflection of the relationship between NO and living organisms at an early stage (stage 1) and signalling functions would be the most evolved and advanced relationship (stage 4). The figure is redrawn from Yamasaki (2004).

ing flooding. Under such conditions, biochemical reactions of NO should be distinguished from those in aerobic conditions. One obvious effect could be a stabilization of NO that ensures its longer action. In the presence of O_2 , NO can be spontaneously oxidized to NO_2 , a major degradation route for NO in the air. Alternatively, NO may rapidly be converted to $ONOO^-$, if an O_2^- generating site is close (Yamasaki 2000). Thus, hypoxic (or anoxic) conditions may enable NO to be a long-distance signal transmitter without causing any oxidative damage of the cells (Stöhr & Ullrich 2002; Dordas, Rivoal & Hill 2003; Stoimenova *et al.* 2003). Therefore, we should pay more attention to the oxygen availability in a NO signalling system.

THE ONS HYPOTHESIS: TO UNIFY THE COMPLEX WORLD

In the past 50 years, we have established oxygen biology that explains how our body can produce energy and how plants assimilate carbon dioxide while producing oxygen, namely, the bioenergetics in respiration and photosynthesis – the main avenue in biochemistry until the 1970s. Since the discovery of oxygen toxicity (McCord & Fridovich 1969), we have learned that energy conversion with oxygen is a risky business and the living organisms presently dominat-

ing on the earth had to overcome the dark side of oxygen, namely the ROS problems. Indeed, oxygen is still lethal for primitive living organisms such as anaerobic bacteria. Furthermore, vertebrates (and higher plants) actively produce ROS in the cells to kill biological invaders. Looking at the history, it appears that NO research has been following the research history of oxygen (or ROS): bioenergetics (nitrate respiration or nitrification), pathogen response (antimicrobial effects) and signalling function in plants and animals.

The interplay between ROS and RNS is a major cause of the complexity (Yamasaki 2000). Theoretically, there is one more candidate that should be included into the oxygen (ROS) and nitrogen (RNS) worlds – the sulphur world. Similar to the case of oxygen (e.g. H_2O_2) or nitrogen (e.g. NO_2^-), the antimicrobial activity of sulphur has been known for many years, but most of our knowledge comes simply from experience. Sulphur dioxide (SO_2) fumigation has been widely used in agriculture for sterilization of fruits for example. SO_2 is even toxic for animals probably due to the toxicity of sulphite (Reist, Jenner & Halliwell 1998). Hydrogen sulphide (H_2S) is a well-known toxic gas that comes from active volcano and industrial activities. As with O_2 and NO, H_2S can bind to heme proteins, thereby inhibiting the redox reactions primary to the cytochrome oxidase (COX) in mitochondria (Yamasaki *et al.* 2001; Bailly *et al.*

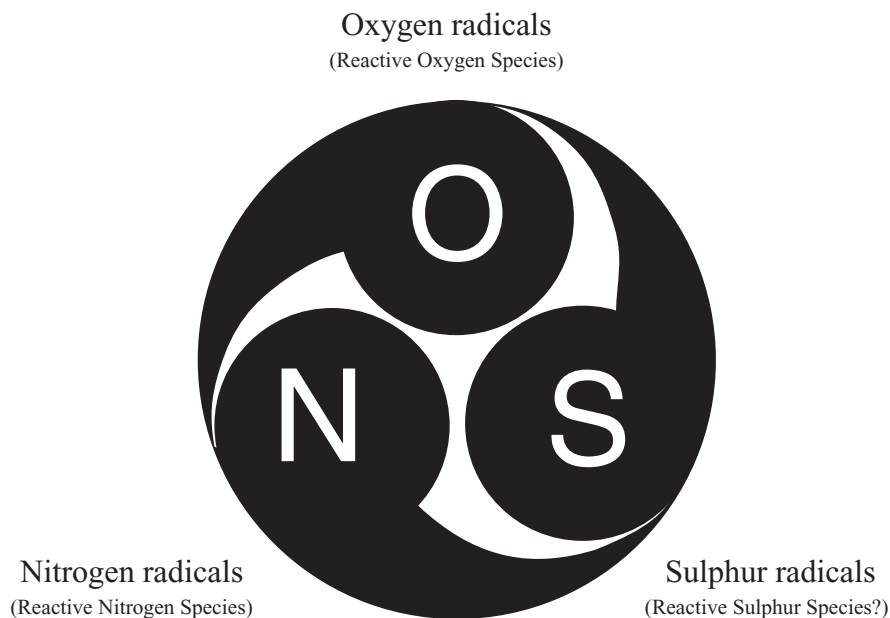


Figure 3. Three dynamic elements in a unity. The 'Mitsudomoe' symbol represents a harmonic relationship between three dynamic elements: oxygen, nitrogen and sulphur radicals in this case. ROS is generated in the chloroplasts as toxic byproducts during the process of CO_2 assimilation (photosynthesis). RNS can be produced as byproducts of nitrate assimilation. Theoretically, there is a possibility that the plant sulphur assimilation process also produces active species of sulphur (RSS, reactive sulphur species). These active molecular species are mutually interactive – a major cause of the complexity of radical biology.

2003). Surprisingly, H_2S is also produced in animal cells and its concentration is high in brain tissue (Abe & Kimura 1996; Kashiba *et al.* 2002). Abe and Kimura have suggested that H_2S might be another gas signal transmitter, equivalent to ROS (H_2O_2) and RNS (NO), for neural communication in vertebrates (Abe & Kimura 1996). For sulphur bacteria that are colonized in anoxic habitats, H_2S is an electron donor for energy transduction: equivalent to NH_4^+ for nitrifying bacteria and H_2O for cyanobacteria. Thus, H_2S shows versatility as similar to O_2 and NO_2^- . This is another refrain of the research history (bioenergetics, antibiotic effects, signalling function).

Figure 3 represents an ultimate feature of the NO world: 'Mitsudomoe' – the Japanese crest of three commas, a representation implying a harmonic unity of three dynamic elements. A similar principle can be seen in Dosha in Ayurvedic philosophy. As mentioned above, research on NO signalling cannot stand without elucidating its interaction with ROS. Although we need to wait for another scientific explosion as in NO research, the biology and biochemistry of reactive sulphur species (RSS) will be highlighted in the near future. Bacterial sulphite reductase flavoprotein (SiRFP) has been proposed to be a prototype of the reducing domain of mammalian NOS (Zemojtel, Wade & Dandekar 2003), a possible linkage in evolution between RNS and RSS.

ROS, RNS and RSS are associated with plant assimilation of carbon, nitrogen and sulphur. The chloroplast is important for these assimilation processes because they require reducing power provided by the photosynthetic electron transport system. The interplay between the three assimilation processes has been a subject of research for improving plant productivity but a unifying mechanism governing multiple systems has yet to be elucidated. Mutual interactions among ROS, RNS and RSS might be a key for clarifying the unknown mechanism: balance sci-

ence for three distinct dynamic elements (ROS, RNS, RSS).

CONCLUDING REMARKS

Research in signalling networks is multi- or inter-disciplinary science in which integration is essential. The application of computer information technology is a powerful way to integrate genomics, proteomics and metabolomics. The concept of systems biology could help to explore the signalling networks (Kitano 2002). However, those trends have not considered the contribution of the labile molecules, such as radicals, nor balance science. Matsumoto *et al.* have suggested that we may have ignored the functional importance of gaseous molecules including NO in protein interactions (Matsumoto *et al.* 2003). Suemastu and coworkers have argued the need for 'gas biology', that covers ROS, RNS and RSS, for signalling research (Kashiba *et al.* 2002). Plant systems are more open to the environment than are those of animals (Fig. 4). Consequently, plant systems may be closely linked to the activities of soil bacteria through changes in NO levels that can vary in response to nitrogen and oxygen availability (Stöhr & Ullrich 2002). To draw a whole picture of NO signalling network in plants, it becomes obvious that we need more active integration of our research, ranging from molecule, cell, tissue, organ, plant–plant, plant–microbe interactions to global issues eventually.

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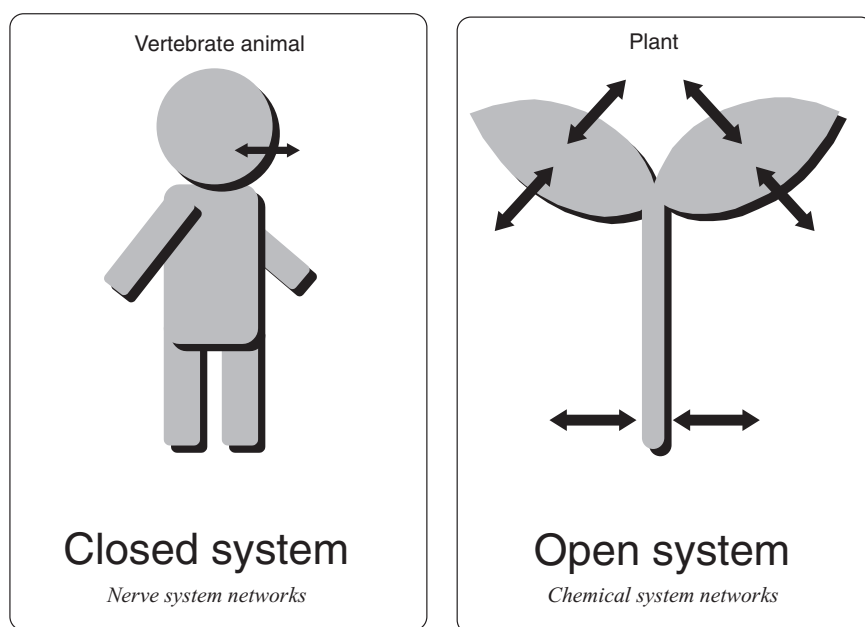


Figure 4. Open or closed system. Plant NO signalling systems should be responsible for exogenous NO emission from the environment as well as endogenous NO production by NOS and NR. This trait provides a fundamental difference that distinguishes plant from animal, namely an 'open system' versus 'closed system' for NO signalling mechanisms. In contrast to plants, NO signalling systems of animals are localized in specific tissues, and are little influenced by environmental NO. This aspect will be important when we argue plant responses in the field.

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